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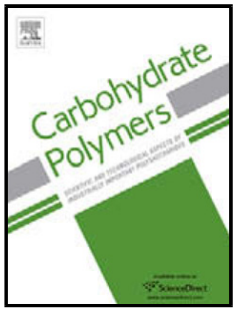

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Efficiency of purification methods on the recovery of exopolysaccharides from fermentation media

Running title - – *Effect on exopolysaccharide recovery from simulant fermentation media*

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Highlights

- MRS medium is the major source of interfering compounds.
- MRS medium was responsible for the introduction of glucose-rich material.
- Lactose may promote the co-precipitation of several medium components.

- YE also contributed with mannoproteins.
- TCA method was shown to be more efficient in recovery of EPS.

Abstract

De-Man Rogosa and Sharpe (MRS) is a complex medium commonly used to obtain exopolysaccharides (EPS) from lactic acid bacteria. However, the various nutrients (carbon and nitrogen sources) of media and supplements added to enhance the bacterial growth and EPS production, may interfere with the purification of the extracts resulting in an over-estimation of the EPS and erroneous structural assignments. The efficiency of trichloroacetic acid (TCA)/pronase and 5-sulfosalicylic acid – SSA methods was evaluated. In addition, the interference of the major MRS broth components as well as lactose was evaluated using xanthan gum as model control EPS. It was concluded that MRS medium is a major source of interfering compounds in the quantification of EPS, mainly glucose-rich material and to a lesser extent mannoproteins from yeast extract. This effect was found to be potentiated by the presence of lactose. TCA/pronase method was shown to be more efficient in eliminating interferents.

Keywords: Exopolysaccharides, trichloroacetic acid/pronase, 5-sulfosalicylic acid, MRS broth, yeast extract, xanthan gum, lactose.

1. Introduction

Several lactic acid bacteria (LAB) strains belonging to the genera *Streptococcus, Lactobacillus, Lactococcus, Leuconostoc* and *Pediococcus* as well as several strains of *Bifidobacterium*, generally used as starter cultures in fermentation processes, are capable of producing exopolysaccharides (EPS) (Bengoa et al., 2018; Inturri et al., 2017; Kanamarlapudi & Muddada, 2017; Saravanan & Shetty, 2016; Song, Jeong, Cha, & Baik, 2013). LAB are relatively fastidious microorganisms in their nutritional requirements for normal growth. Besides a carbon source, LAB require nitrogen, partially in the form of amino acids, several vitamins, growth substances, and minerals. The composition of the

medium used for cultivation of LAB may influence both lactic acid and EPS production. De-Man Rogosa and Sharpe medium (MRS) (De Man, Rogosa, & Sharpe, 1960) which is a complex, nutrient-rich medium based on yeast extract and peptones, has been designed to stimulate the growth of LAB and in the case of *Bifidobacterium* spp. is often supplemented with L-cysteine as an oxygen scavenger (Ashraf & Shah, 2011; Prasanna, Grandison, & Charalampopoulos, 2014). This growth medium is commonly used for the enrichment, cultivation and isolation of culture EPS-producing LAB, either as liquid broth or as agar plates since it is easy to prepare and inexpensive when compared with semi-defined or chemically defined synthetic media, (Polak-Berecka et al., 2015; Yang et al., 2018; Zhou et al., 2016; Leroy & De Vuyst, 2016; Rajoka et al., 2018). Supplements such as carbon and nitrogen sources and mineral salts are frequently added thereto for the purpose of growing the microorganism and increasing the EPS yield (Fukuda et al., 2010; Kimmel & Roberts, 1998; Liu et al., 2016; Petrovici et al., 2017). MRS broth medium contains beef extract (1.0%), peptones (1.0%), and yeast extract (0.5%) which can be recovered together with the EPS (Alhudhud, Humphreys, & Laws, 2014; Ruas-Madiedo & de los Reyes-Gavilán, 2005; Torino, Sesma, & Font De Valdez, 2000). Moreover, the EPS yields may also be overestimated due to the presence of specific carbohydrates, either from the media or often added with the intention of stimulating bacterial growth, e.g. lactose (Cerning et al., 1994; Rimada & Abraham, 2003; Audy, Labrie, Roy, & LaPointe, 2010), which are not completely eliminated during EPS recovery and therefore are included in the EPS quantification. The choice of an adequate EPS production medium is of great importance for the successful recovery of EPS, since besides influencing the production yield, it can also contain components that may not be efficiently eliminated during the isolation process and may interfere with detection and quantification methods (Nguyen et al., 2018; Behare et al., 2009; Leroy & De Vuyst, 2016).

Thus, the selection of the appropriate methodology for the isolation and purification of EPS is crucial in order to obtain maximum yields with high purity (Leroy & De Vuyst, 2016; Mende, Rohm, & Jaros, 2016). The majority of the reported procedures comprehend the cells removal by centrifugation, followed by the precipitation of EPS by the addition of a water miscible polar organic solvent; precipitation with chilled ethanol is largely applied to concentrate the EPS (Othman, Din, Mohammad, Rosli, & Sarmidi, 2018; Ricciardi et al., 2002; Leroy & De Vuyst, 2016; Rani, Anandharaj, & David Ravindran, 2018). When more complex media are used, additional pre-treatment

steps are required to reduce the presence of proteins in the final EPS-rich extracts (Pachekrepapol, Lucey, Gong, Naran, & Azadi, 2017; Polak-Berecka et al., 2015). The most frequent procedure used for protein elimination involves trichloroacetic acid (TCA) precipitation (final concentrations ranges are usually between 4 and 20%), digestion with proteases (e.g. protease such as pronase E, proteinase K or flavourzyme) or sequential combination of both, and removal by centrifugation followed by EPS concentration by ethanol precipitation (Dinic et al., 2018; Marshall et al., 2001; Wang, Zhao, Yang, Zhao, & Yang, 2015; Yang et al., 2018). Other protein precipitating agents (Greenberg & Shipe, 1979) such as 12 M HCl (Enikeev, 2012; Khanal & Lucey, 2017) 5-sulfosalycilic acid (SSA) (Pintado, Pintado, & F. Malcata, 1999a; Pintado, Pintado, & Malcata, 1999b) or ammonium sulphate (Peng, Han, Liu, & Zhou, 2016), have also been successfully introduced. After the EPS precipitation, the last step of EPS purification generally includes the resuspension of resulting material in deionized water and its dialysis against water for 1 to 7d at 4°C to remove low-molecular-mass contaminating carbohydrates that may be present in the material, and finally the resulting EPS is lyophilized (Abid et al., 2018; Benit & Roslin, 2018; London et al., 2014). The amount and composition of the isolated EPS have been shown to vary with the type of adopted approach (Mende et al., 2016; Miao, 2015), and a systematic assessment of the most effective methods for removing media contaminants remains to be done (Nguyen et al., 2018).

Many published works have highlight the problem of interfering components in the quantification of EPS usually leading to a false increase in the final yield of EPS. However, the information on the role of each contaminant component is still scant and scattered.

This study aims to evaluate the efficiency of the two purification methods, TCA/pronase (Method I) and SSA (Method II), in recovering EPS from MRS broth medium. A systematic study on the identification of the major interfering components in a MRS growth medium was done, and how these components can interfere individually or combined in the EPS quantification was further evaluated. Attempts were also made to determine the contribution of possible carbohydrates contaminants sources, namely yeast extract and lactose. A large number of published works involve media containing lactose, as milk, cheese whey or synthetic media with added lactose to increase EPS yields by stimulating LAB growth and EPS production (Audy, Labrie, Roy, & LaPointe, 2010; Polak-Berecka et al., 2015; Ayala-Hernández, Hassan, Goff, de Orduña, & Corredig, 2008) which are often used in the manufacture of fermented milk products in the dairy

industry. In studies with *Xanthomonas campestris* although glucose and sucrose are the most frequently used carbon sources, other lactose-containing culture media are reported too, namely acid whey (Charles & Radjai, 1977) and cheese whey (Silva et al., 2009). Therefore, lactose was chosen for the present study because it can be found as a contaminant of the final extracts (Rimada & Abraham, 2003) given its uncomplete elimination during EPS recovery and therefore its inclusion in the EPS quantification.

For this purpose, three model solutions were used: lactose, yeast extract and xanthan gum that is produced from *Xanthomonas campestris* pv.; the most popular commercially accepted microbial EPS, which was elected as a model control EPS. The effect of both purification methods was evaluated with the MRS broth medium, with the individual model solutions and with both combined.

2. Materials and methods

2.1. Model solutions

Eleven model solutions were prepared: (i) 0.1% (*w/v*) xanthan gum (X); (technical grade) purchased from Jungbunzlauer (Wien, Áustria);(ii) 1.5% (*w/v*) lactose (L) (monohydrate) purchased from Merck (Darmstadt, Germany); (iii) 1.5% (*w/v*) lactose with 0.1 % (*w/v*) xanthan gum (X+L); (iv) 0,5% (*w/v*) Yeast Extract (YE) purchased from Biokar (Diagnostics, Beauvair, France) (v) YE containing 1.5% (*w/v*) lactose (YE+L); (vi) YE containing 0.1% (*w/v*) xanthan gum (YE+X); and (vii) YE containing 1.5% (*w/v*) lactose with 0.1% (*w/v*) xanthan gum (YE+X+L).(viii) MRS broth medium (MRS) purchased from Biokar (Diagnostics, Beauvair, France); (ix) MRS broth medium containing 1.5% (*w/v*) lactose (MRS+L); (x) MRS broth medium containing 0.1% (*w/v*) xanthan gum (MRS+X); (xi) MRS broth medium containing 1.5% (*w/v*) lactose with 0.1% (*w/v*) xanthan gum (MRS+X+L); The full composition of the model solutions is presented in Table 1.

Table 1 – Composition of the eleven model solutions.

Model solutions	Composition (g/L)										
	X	**L**	**X+L**	**YE**	**YE+L**	**YE+X**	**YE+X+L**	**MRS**	**MRS+L**	**MRS+X**	**MRS+X+L**
Xanthan	1.00	–	1.00	–	–	1.00	1.00	–	–	1.00	1.00
Lactose	–	15.00	15.00	–	15.00	–	15.00	–	15.00	–	15.00
Dextrose or glucose	–	–	–	–	–	–	–	20.00	20.00	20.00	20.00
Tween 80	–	–	–	–	–	–	–	1.08	1.08	1.08	1.08
Ammonium citrate	–	–	–	–	–	–	–	2.00	2.00	2.00	2.00
Sodium acetate	–	–	–	–	–	–	–	5.00	5.00	5.00	5.00
$MgSO_4.7H_2O$	–	–	–	–	–	–	–	0.20	0.20	0.20	0.20
$MnSO_4$	–	–	–	–	–	–	–	0.05	0.05	0.05	0.05
K_2HPO_4	–	–	–	–	–	–	–	2.00	2.00	2.00	2.00
Beef extract	–	–	–	–	–	–	–	10.00	10.00	10.00	10.00
Yeast extract	–	–		5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00
Proteose peptone	–	–	–	–	–	–	–	10.00	10.00	10.00	10.00

2.2.EPS purification methods - Precipitation of proteins and isolation of EPS

All model solutions were treated by two precipitation methods under study. Method I *(TCA/pronase)* - includes protein precipitation with trichloroacetic acid (TCA) in combination with digestion by a protease (Pronase E) and method II (SSA) - includes protein precipitation with 5-sulfosalicylic acid (SSA) as described below.

For EPS control (X control), the xanthan gum model solution (X) was also prepared for further analysis without any protein precipitation pre-treatment. An aqueous solution of 0.1 %(w/v) xanthan gum was precipitated with three volumes of cold ethanol and was held at 4 ºC overnight and submitted to centrifugation (at 2665 x*g*, for 20 min, at 4 ºC). Afterwards, the pellets and supernatants (which are usually rejected) were recovered and stored at 20 ºC until further analysis. The ethanol precipitation allowed the recovery of the compounds insoluble in ethanol (pellet) separately from those that remained in the supernatant. Both, the pellet and the supernatant, were dialysed using a membrane with a cut-off of 12-14 kDa, freeze-dried and submitted to sugars analysis after acid hydrolysis.

All model solutions were carried out in quadruplicate and the results were expressed as mean values and associated standard deviations.

2.2.1. Method I - (TCA/pronase)

This isolation procedure was based on that described by (Gancel & Novel, 1994 and Kimmel & Roberts, 1998) with some modifications. For all model solutions (10.0 mL), the material was first digested with 100 µl of pronase E (5% w/v) at 37 ºC for 1 hour in a water bath shaker and then precipitated by the addition of one volume of 20% (w/v) TCA and incubation for 1 h at 20 ºC. The insoluble material was then recovered by centrifugation at 2665 x*g*, for 20 min, at 4 ºC and discarded. The material remaining in the supernatants was recovered by precipitation with three volumes of cold ethanol, standing at 4 ºC overnight. Subsequently, the precipitated material was isolated by centrifugation at 2665 x*g*, for 20 min, at 4 ºC. The pellets so obtained were further dispersed in distilled water, and both, pellets and supernatants, were dialysed using a membrane with a cut-off of 12-14 kDa, freeze-dried and subjected to sugars analysis after acid hydrolysis as described in section 2.3 below.

2.2.2. Method II – (SSA)

The SSA purification method included an initial protein precipitation step where all model solutions (10.0 mL) were treated with 5-sulfosalicylic acid according to that described by (Pintado et al., 1999a with some modifications). It consisted of addition of 2% (w/w) 5-sulfosalicylic acid followed by incubation at 4 °C for 1 h. The subsequent steps of separation of the soluble material and its recovery by precipitation with ethanol were performed as in method I. The pellets obtained were further dispersed in distilled water, and both, pellets and supernatants, were dialysed using a membrane with a cut-off of 12-14 kDa, freeze-dried and subjected to sugars analysis after acid hydrolysis as described in section 2.3 below.

2.3. Sugars analysis

Neutral sugars were released by Saeman hydrolysis (Selvendran, March, & Ring, 1979) and analysed as their alditol acetates by gas chromatography (Blakeney, Harris, Henry, & Stone, 1983; Harris, Blakeney, Henry, & Stone, 1988) using a Carlo Erba 6000 apparatus with a split injector (split ratio 1:60) and a flame ionisation detector as described by (Mafra et al., 2001). Samples were prepared in triplicate by hydrolysis in 0.2 mL of 72% H_2SO_4 for 3 h at 20 ºC, followed by dilution to 1 M H_2SO_4 and hydrolysis for 2.5 h at 100 ºC. The column was a DB- 225 (J & W, USA), 30 m, 0.25 mm I.D., and film thickness of 0.15 µm and the oven temperature program was: 220 ºC for 5 min and then the temperature was raised at 20 ºC/min to 230 ºC and maintained at this temperature for another 6 min. The flow rate of the carrier gas (H_2) was set at 1 mL min^{-1} at 220 ºC. The injector and the flame ionisation detector (FID) were maintained at 220 and 230 ºC, respectively. The hydrolysis of all samples was done in triplicate and each one was injected twice. Results with less than 5% variability in the major component cell wall sugars were obtained.

Uronic acids were determined colorimetrically by a modification (Coimbra, Delgadillo, Waldron, & Selvendran, 1996) of the method of (Blumenkrantz & Asboe-Hansen, 1973). Calibration was made with D-galacturonic acid.

2.4. Statistical analyses

One-way analyses of variance (ANOVA) were conducted to assess the effects (monitored by total yield of EPS and by monosaccharide composition) of the two EPS purification methods upon the different model solutions (with and without xanthan gum addition) either to the pellets or to the supernatants and pairwise comparisons of mean values (following Tukey's test) at the 5% significance level were performed. Non-parametric test – Wilcoxon-Mann-Whitney (two independent samples) and Kruskal-Wallis (k independent samples) were then employed to find out whether such differences, between the two protein precipitation methods for different dependent variables in study, were in turn significant. All tests were performed using SPSS software for Windows, v. 16.0 (SPSS, Chicago IL, USA).

3. Results and discussion

This study compares the performance of two methods (methods I and II) for the isolation of xanthan gum, used as model control EPS, from complex fermentation MRS medium. The degree of contamination with yeast extract and lactose was also estimated. Envisaging these goals, model solutions of xanthan gum (X), lactose (L), yeast extract (YE) and MRS broth medium (MRS), and combinations of these components, were subjected to the two EPS purification methods

3.1. Xanthan gum model control solution

All the 10 mg of initial material used (0.1 %(w/v) xanthan gum solution) was recovered in the pellets and none was found in the supernatant. The sugar analysis performed revealed 4.2 mg as carbohydrates, yielding 42% of sugars, comprehending mannose, glucose and uronic acid (Man:Glc:UA) in a proportion of 0.8:1.3:1.0 (see X control in Table 2).

Table 2 – Monosaccharide composition and total sugars present in the ethanol precipitated (pellet) and ethanol soluble fractions (supernatant) after treatment with method I (TCA/pronase) or method II (SSA) used for purification of EPS.

Recovery EPS method	Model solutions with xanthan	*Pellets*				
		Monosaccharide composition (mg)				Total sugars (mg)
		Mannose	Galactose	Glucose	Uronic acid	
X control		1.12 ± 0.07	0.00 ± 0.00	1.74 ± 0.18	1.35 ± 0.06	4.2 ± 0.13
Method I (TCA/pronase)	X	1.60 ± 0.60 ^{abc}	0.07 ± 0.02 ^{abcd}	2.19 ± 0.47 ^{a}	1.63 ± 0.31 ^{abc}	5.49 ± 1.09 ^{b}
	L	0.00 ±0.00 ^{A}	0.00 ± 0.00 ^{A}	0.00 ±0.00 ^{A}	0.00 ± 0.00 ^{A}	0.00 ± 0.00 ^{A}
	X+L	1.64 ± 0.42 ^{abcd}	0.07 ± 0.03 ^{abcd}	2.04 ± 0.41 ^{a}	1.63 ± 0.26 ^{abc}	5.37 ± 0.62 ^{ab}
	YE	0.30 ± 0.04 ^{BC}	0.02 ± 0.00 ^{AB}	0.04 ± 0.01 ^{A}	0.04 ± 0.01 ^{A}	0.40 ± 0.05 ^{A}
	YE+L	0.20 ± 0.02 ^{AB}	0.00 ± 0.00 ^{A}	0.03 ± 0.01 ^{A}	0.03 ± 0.01 ^{A}	0.26 ± 0.03 ^{A}
	YE+X	2.11 ± 0.54 ^{bcd}	0.04 ± 0.01 ^{abc}	1.76 ± 0.52 ^{a}	1.84 ± 0.16 ^{cd}	5.74 ± 0.107 ^{b}
	YE+X+L	1.12 ± 0.16 ^{a}	0.04 ± 0.01 ^{abc}	1.17 ± 0.07 ^{a}	1.01 ± 0.33 ^{ab}	3.34 ± 0.53 ^{a}
	MRS	0.37 ± 0.11 ^{BCD}	0.05 ± 0.01 ^{B}	1.43 ± 0.32 ^{B}	0.23 ± 0.05 ^{B}	2.07 ± 0.39 ^{B}
	MRS+L	0.60 ± 0.11 ^{DE}	0.11 ± 0.02 ^{C}	2.97 ± 0.46 ^{C}	0.29 ± 0.03 ^{B}	3.97 ± 0.53 ^{C}
	MRS+X	1.20 ± 0.12 ^{a}	0.11 ± 0.06 ^{cde}	3.62 ± 0.56 ^{b}	1.19 ± 0.12 ^{ab}	6.12 ± 0.74 ^{bc}
	MRS+X+L	2.39 ± 0.48 ^{cd}	0.12 ± 0.06 ^{de}	5.21 ± 1.14 ^{c}	2.11 ± 0.4 ^{d}	9.83± 1.70 ^{d}
Method II (SSA)	X	1.35 ± 0.37 ^{ab}	0.02 ± 0.00 ^{ab}	1.59 ± 0.40 ^{a}	1.44 ± 0.25 ^{abc}	4.40 ± 1.01 ^{ab}
	L	0.00 ±0.00 ^{A}	0.00 ± 0.00 ^{A}	0.00 ± 0.00 ^{A}	0.00 ±0.00 ^{A}	0.00 ±0.00 ^{A}
	X+L	1.11 ± 0.16 ^{a}	0.04 ± 0.01 ^{abc}	1.34 ± 0.15 ^{a}	0.96 ± 0.19 ^{a}	3.28 ± 0.39 ^{a}
	YE	0.41 ± 0.03 ^{BCD}	0.02 ± 0.00 ^{AB}	0.08 ± 0.01 ^{A}	0.04 ± 0.01 ^{A}	0.55 ± 0.04 ^{A}
	YE+L	0.45 ± 0.06 ^{CD}	0.00 ± 0.00 ^{A}	0.08 ± 0.02 ^{A}	0.08 ± 0.02 ^{A}	0.61 ± 0.10 ^{A}
	YE+X	2.14 ± 0.19 ^{bcd}	0.00 ± 0.00 ^{a}	1.94 ± 0.35 ^{a}	2.02 ± 0.24 ^{cd}	6.10 ± 0.50 ^{bc}
	YE+X+L	1.72 ± 0.21 ^{abcd}	0.02 ± 0.00 ^{ab}	1.83 ± 0.19 ^{a}	1.40 ± 0.34 ^{abc}	4.97 ± 0.19 ^{ab}
	MRS	0.92 ± 0.19 ^{F}	0.12 ± 0.02 ^{C}	5.54 ± 0.51 ^{D}	0.43 ± 0.05 ^{C}	7.01 ± 0.65 ^{D}
	MRS+L	0.76 ± 0.17 ^{EF}	0.19 ± 0.03 ^{D}	5.13 ± 0.70 ^{D}	0.42 ± 0.08 ^{C}	6.50 ± 0.94 ^{D}

	MRS+X	1.52 ± 0.20 ^{ab}	0.09 ± 0.01 ^{bcde}	5.00 ± 0.27 ^{c}	1.49 ± 0.11 ^{abcd}	8.10 ± 0.55 ^{cd}
	MRS+X+L	2.47 ± 0.45 ^{d}	0.15 ± 0.04 ^{e}	7.18 ± 0.48 ^{d}	2.89 ± 0.26 ^{e}	12.68 ± 0.56 ^{e}

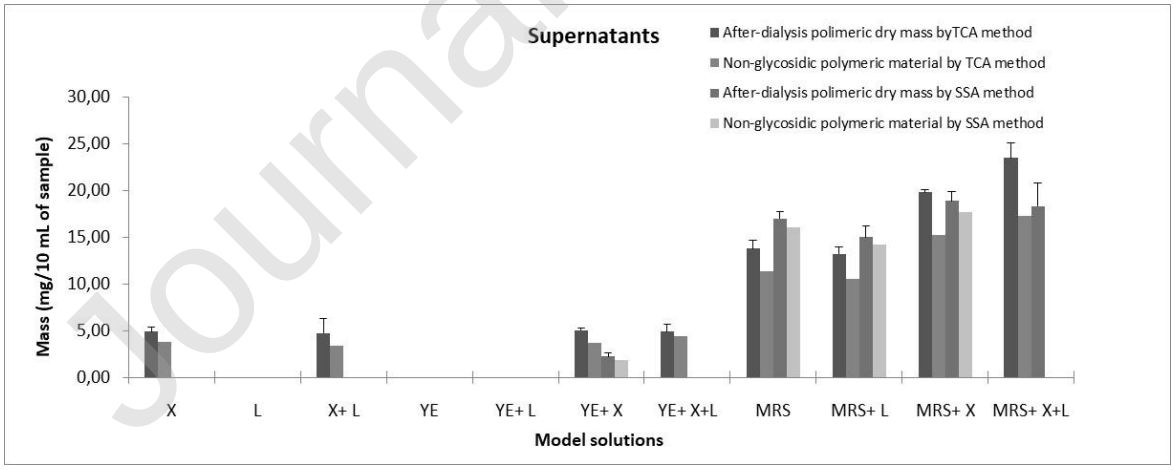
The values are expressed as means ± SD obtained from four replicate measurements.
$^{a-f}$ Means in columns without common letters are significantly different (p<0.05; n=48); $^{A-F}$ Means in columns without common letters are significantly different (p<0.05; n=40)

Abbreviations: X - Xanthan gum solution; L - Lactose solution; X+L – Lactose and xanthan gum solution; YE - Yeast Extract solution; YE+L - YE and lactose solution; YE+X - Yeast Extract solution and xanthan gum solution; YE+X+L – Yeast Extract solution, lactose and xanthan gum solution; MRS - MRS broth medium; MRS+L - MRS broth medium and lactose solution; MRS+X - MRS broth medium and xanthan gum solution; MRS+X+L - MRS broth medium, lactose and xanthan gum solution; method I (TCA/pronase) or method II (SSA) - recovery EPS methods.

As the structure of xanthan consists of pentasaccharide subunits with a β-1,4-linked D-Glc backbone exhibiting as side chains the trisaccharide [β-D-Man-(1→4)-β-D-GlcA-(1→2)-α-D-Man-(→] linked to the O-3 of alternate Glc residues in the backbone (Jansson, Kenne, & Lindberg, 1975), the theoretical proportion should be 2:2:1. Thus, the amounts of Man and Glc found in the commercial xanthan may have variations that could result in slightly different proportions of the sugars components of the repetitive structure, as was observed for other polysaccharides such as gellan gum (Gonçalves et al., 2009). It is also possible that some underestimation occurred as a result of the non-quantification of neutral sugars esterified with organic acids, known to occur in this polysaccharide (Sutherland, 1981; Stankowski, Mueller, & Zeller, 1993) as well as the difficulty in the hydrolysis of aldobiouronic acids (Ferreira et al., 2011). Because all samples were analysed under similar conditions, this bias was taken into account, not influencing the relative content of EPS to be recovered from the different model solutions.

The sugars composition obtained when X model solution was treated with TCA/pronase or SSA was similar to the one obtained for the control and comparable between them ($p>0.05$). However, a small amount of polymeric material was recovered in the supernatant obtained by the TCA/pronase method, but not in the supernatant treated with SSA (Fig. 1a).

a)



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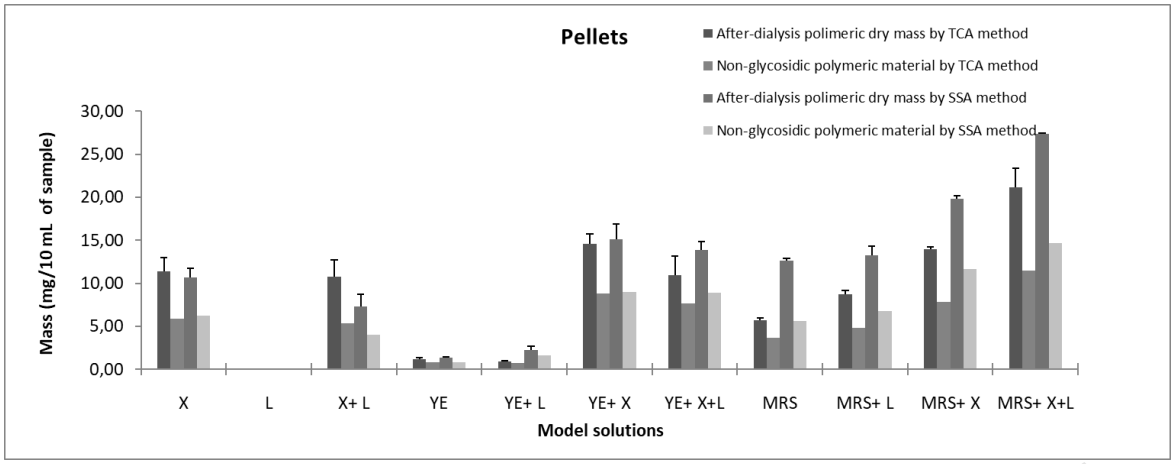

Figure 1 – Content for polymeric dry mass and non-glycosidic polymeric material (difference between the polymeric dry mass and total sugars) for recovery of EPS by TCA/pronase and SSA. **a)** In the supernatants; **b)** In the pellets.

This material was mostly of non-glycosidic nature (3.81 mg/10 mL of sample); only residual amounts of sugars (11%), with a monosaccharide composition similar to the pellets, were present (Table 2). This seems to be due to incomplete precipitation by TCA allowing polymeric material to be subsequently precipitated by ethanol coupled with some sugars from the yeast extract present in the basal media used. Although the results of the pellets from the two purification methods do not show significant differences, apparently, the SSA method was more efficient than the TCA/pronase method in removing non-glycosidic interferents from the EPS alone (5.88 mg/10 mL of sample for TCA/pronase and 6.23 mg/10 mL of sample for SSA method) and allowing its total recovery by ethanol precipitation (Table 2).

3.2. Effect of lactose on the recovery of xanthan gum

In order to assess the influence of lactose on contamination of EPS recovery, model solutions containing only lactose (L) were treated with TCA/pronase or SSA prior to precipitation with ethanol and dialysis. As observed in Figure 1, lactose was efficiently removed in both cases.

With the addition of lactose to xanthan (X+L), the results of TCA/pronase and SSA methods were comparable and did not differ from those obtained for the xanthan solution alone ($p>0.05$; Table 2 and Fig. 1). Moreover, the sugar analysis of the isolated fractions did not reveal traces of lactose, thus reinforcing the idea that lactose was totally eliminated during the purification. Noteworthy, even though not statistically significant, the SSA method showed lower polymeric dry mass and total sugars in the pellets (Fig. 1b) due to a decrease in UA (Table 2). The pellets obtained with the TCA/pronase method registered Man:Glc:UA in a proportion of 1.0:1.2:1.0, which is consistent with the results obtained for xanthan gum using the same method (1.0:1.3:1.0).

3.3. Effect of yeast extract on the recovery of xanthan gum

3.3.1. Yeast extract (YE) and yeast extract + lactose (YE+L) model solutions

The application of TCA/pronase and SSA purification methods to YE model solutions showed a similar ($p>0.05$; Fig. 1) removal of approximately 97% of the initial material. Mannose, which is derived from mannoproteins, abundantly present in yeast extracts (Quirós, Gonzalez-Ramos, Tabera, & Gonzalez, 2010; Vaningelgem et al., 2004), made up approximately 75% of each sugar fraction. Residual amounts of Gal, Glc and UA, also components of yeast cell walls (Pinto, Coelho, Nunes, Brandão, & Coimbra, 2015), were detected in the recovered pellets. This demonstrates that both methods fail to remove all the YE components, including sugars-rich polymeric material, which may constitute a source of EPS contamination.

The addition of lactose to YE showed similar results to those observed for YE alone ($p>0.05$). Nevertheless, it is important to highlight that the SSA method enabled slightly higher contents for polymeric dry mass in the pellets when compared to YE alone (Fig. 1). This suggests that lactose may interfere with the capability of the SSA to remove polymeric material from YE, in particular mannoproteins.

3.3.2. Yeast Extract + xanthan gum (YE+X) and yeast extract + xanthan gum + lactose (YE+X+L) model solutions

Independently of the purification method employed, the fractions isolated from YE+X model solutions consistently showed higher polymeric yield than the 10 mg of xanthan gum added (Fig. 1). Polymeric material was also found in the supernatants obtained with SSA precipitation, whereas no mass had been found for YE and X model solutions. In addition, the pellets from SSA purification also showed higher sugar content than those obtained by the sum of individual X and YE model solutions (Table 2). This was mostly due to the much higher increase in mannose, thus suggesting that the presence of the EPS contributes to an increase of the co-precipitation of YE mannoproteins. A higher mannose content was also observed in the pellets of the TCA/pronase method, even though not statistically significant, when compared with X model solution alone. These results demonstrate that YE contributes to the contamination of EPS-rich extracts; namely, it is responsible for the introduction of mannose that can be erroneously estimated as part of the EPS moiety. The SSA purification method was revealed to be less efficient in the removal of these contaminants.

When lactose was added to YE+X model solution (YE+X+L), the polymeric mass and total sugars (Fig. 1b, Table 2) in pellets from both purification methods were significantly ($p<0.05$) lower than those of YE+X for the TCA/pronase purification method. A decrease in the total sugars was also observed, resulting mostly from a decrease in mannose and uronic acids contents. Noteworthy is the fact that the presence of lactose also eliminated polymeric material from the ethanol supernatants obtained from the SSA method and significantly reduced the amount of sugars in the supernatants of the TCA/pronase purification method. In addition, sugar analysis did not reveal an increase in galactose when comparing with the extracts lacking lactose, thus demonstrating that all lactose was eliminated by both methods. These results further suggest that the presence of lactose prevented contaminations with mannoproteins from YE and highlights the lower efficiency of the SSA purification method when compared to the TCA/pronase counterpart.

In summary, the experiments with YE+X and YE+X+L model solution showed that lactose was easily eliminated from the medium and had a positive effect in preventing contaminations with mannoproteins and UA-rich material. The TCA/pronase purification method was more efficient than the SSA method in the isolation of xanthan from complex model solutions containing yeast extract and lactose.

3.4. Effect of the MRS broth medium in the recovery of EPS

As previously mentioned, MRS broth is the most commonly used laboratory medium to grow LAB and promote the production of EPS. The composition of MRS broth listed in Table 1 reveals considerable amounts of polymeric extracts that are often found as contaminants of EPS (Kimmel & Roberts, 1998; Vaningelgem et al., 2004). As shown, YE is a major source of mannoproteins and other glycans, as well as of non-glycosidic material (Table 2 and Fig. 1). Furthermore, beef extract, which is a complex nutrient also present, has high glycogen content and has been reported as a source of contamination which may interfere with EPS determination, as well as peptones that may be responsible mainly for the introduction of proteinaceous material (Kimmel & Roberts, 1998).To evaluate the extent of the possible contamination of MRS components and the performance of the purification methods under study to purify the EPS from MRS medium, experiments were carried out using model solutions containing MRS broth medium and xanthan gum. The influence of lactose was also determined.

3.4.1. MRS broth medium (MRS) and MRS + lactose (MRS+L) model solutions

The pellets and supernatants from the MRS broth showed considerable amounts of polymeric material, independently of the method used (Fig. 1). Important to note is the fact that the recovered material was significantly higher in the supernatants than in the pellets and mainly of non-glycosidic nature. This shows that both purification methods failed to remove all the polymeric material present in the medium. However, part of this drawback was overcome by ethanol precipitation, as a large proportion of the contaminant material, but not all, remained soluble in the ethanol solution. The treatment with TCA/pronase was found to be more efficient than the SSA counterpart.

The pellets and supernatants also exhibited considerable amounts of polymeric sugars, which were significantly higher in the pellets from the SSA method (Table 2). The main sugar in these fractions was Glc, possibly glycogen derived from beef extract, followed by lower amounts of Man, Gal and UA, possibly from yeast extract. The pellets obtained from the TCA/pronase purification method also presented mannose contents similar to those observed in YE model solutions, contrarily to the SSA method, which recovered approximately three times more mannose. Thus, the TCA/pronase was shown to

be more efficient in the elimination of both non-glycosidic and glycosidic polymeric material, in particular of Glc- and Man-rich nature. These results also demonstrate that MRS may significantly contribute to the impurity of the EPS-rich extracts by introducing mainly Glc-rich polymers and mannoproteins beyond other non-glycosidic compounds.

The addition of lactose to MRS promoted an increase of polymeric material in the pellets when compared with MRS alone, which was more pronounced when the TCA/pronase purification method was used (Fig. 1b). Higher total sugar amounts were also observed in the pellets obtained with the TCA/pronase method due to an increase in Glc, and to a less extent Man and Gal (Table 2). Such observations suggest that lactose contributes to increase the amount of MRS contaminants in the pellets when using the TCA/pronase method. Moreover, both purification methods showed pellets with increased content of Gal in relation to MRS alone, thus denoting residual amounts of lactose. The supernatants from the TCA/pronase method did not differ from those of MRS alone in what concerns the parameters under study, while those from the SSA method showed a slightly lower amount of polymeric material. Despite these observations, the TCA/pronase method was more efficient in the removal of MRS contaminants when compared to the SSA method, although both were negatively affected by the presence of lactose.

3.4.2. MRS + xanthan gum (MRS+X) and MRS + xanthan gum + lactose (MRS+X+L) model solutions

The combination of MRS with xanthan gum significantly increased the levels of polymeric material in the pellets and supernatants from both purification methods when compared to those obtained for MRS or xanthan gum alone (Fig. 1 and Table 2). This resulted in a higher recovery of material of both non-glycosidic and glycosidic nature. Still, the total mass and sugars of MRS+X model solutions were lower than the sum of values reported for the MRS and X model solutions, suggesting a lower contamination level of the final sample. The sugars composition of the pellets showed enrichment in Man, Glc, and UA for both methods, when compared to those from MRS alone, thus confirming the recovery of the EPS. However, the increase in Glc beyond the proportions found in xanthan denoted contamination with MRS components. It further highlights that the EPS contributes to the co-precipitation of Glc-rich material. The levels of Glc contamination were more pronounced for the SSA method, showing more Glc than that obtained with the TCA/pronase method, explaining its higher sugars percentage. Conversely, the supernatants

revealed significantly higher sugars content with the TCA/pronase method, mostly due to Glc. This reveals that the use of TCA/pronase effectively reduces contamination in the pellet by inducing the solubility of Glc-rich material. This may be a result of TCA partial hydrolysis of glycogen and pronase digestion of glycoproteins containing Glc (Dodds, Seipert, Clowers, German, & Lebrilla, 2008). In summary, MRS+X model solutions showed that the EPS promoted an increase in the levels of non-glycosidic contaminants in the final extract, mostly composed of Glc-rich material derived from MRS. The high polymeric nature of the EPS has been found to have encapsulating properties or simply by adsorption (Zeng et al., 2016; Pen et al., 2015; More, Yadav, Yan, Tyagi, & Surampalli, 2014), which may contribute to this fact. Still, these effects were less pronounced upon treatment with TCA/pronase.

The addition of lactose to MRS+X solution increased the amount of polymeric material recovered by TCA/pronase and SSA purification methods when compared with either MRS+X or MRS+L model solutions, exceeding the mass of 10 mg of xanthan gum present (Fig.1). This increase was more pronounced in the pellets than in the supernatants. The mass values of both purification methods increased significantly ($p<0.05$) although this increase was more pronounced for the SSA method, resulting from higher amounts of non-glycosylated contaminants as well as from carbohydrates. In particular, the pellets from MRS+X+L solution exhibited higher Glc content and, to a lesser extent, more Man and UA than those from MRS+L and MRS+X model solutions (Table 2). Also, as described for the other MRS containing solutions, the SSA extracts presented more sugars than those obtained with TCA/pronase, confirming the higher levels of contamination obtained by SSA. This observation was reinforced with the analysis of the supernatants, which confirmed the efficiency of TCA/pronase in removing more Glc-rich material from the pellets by promoting its solubility in ethanol solutions.

The amount of Gal in the pellets did not increase with the addition of lactose to MRS+X model solutions, reinforcing the hypothesis that lactose is not a contaminant of EPS. However, the data demonstrated that lactose promoted the co-precipitation of several contaminants, including Glc-rich material. Moreover, the increase in Man and UA beyond the estimated value obtained with X model solutions further demonstrated that mannoproteins and other polymers were also being co-precipitated. Nonetheless, the exact origin of the UA excess reported remains to be unequivocally determined. The observations do suggest it to originate from YE in YE+X model solutions.

Globally, the TCA/pronase purification method remained more efficient than the SSA counterpart in reducing the levels of medium contaminants of EPS-rich extracts recovered from MRS broth. However, the presence of lactose significantly contributed to increase the levels of contamination. These results contrast with the observations from YE+X+L where, apparently, lactose contributed to reduce the levels of contamination of the EPS-rich extracts.

4. Conclusion

The results reveal that MRS medium, commonly used to grow lactic acid bacteria and produce EPS, does introduce both non-glycosidic and glycosidic contaminants in EPS-rich extracts. In particular, significant amounts of Glc-rich material were detected most likely originating from glycogen in beef extract. YE also contributes with mannoproteins. However, the levels of contamination with this material were considerably lower than with MRS that contains the same percentage of YE which highlights the influence of the other medium components. In addition, xanthan gum recovered from MRS medium yielded considerable amounts of UA whose origin remains to be fully determined. It was also found that lactose, frequently added to the culture medium to enhance EPS production, is not a contaminant of the EPS. Nevertheless, lactose promotes the co-precipitation of several medium components, thereby decreasing the purity of the EPS. The TCA/pronase method is more efficient than the SSA counterpart, although significant amounts of contaminants are still present in the final extracts. These observations explain the high percentage of non-glycosidic material found in xanthan gum commercial extracts (58%) and reinforce the need for caution in the interpretation of results during EPS purification and yield determination.

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